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14. ABSTRACT

Breast cancer brain metastasis (BCBM) is devastating and increasing in frequency, however, BCBM mechanisms are understudied and remain largely unknown. Further, although notions that circulating tumor cells (CTCs) acting as "seeds" of intractable metastasis are established, virtually nothing is known about the properties and biomarkers of BCBM colonizing CTCs. We hypothesized that *Notch1 and HPSE are novel CTC biomarkers to predict the presence of primary breast cancer brain metastasis; and they can be potential therapeutic targets to prevent secondary BCBM.* Accordingly, we isolated CTC subsets expressing Notch1/HPSE combinations from metastatic HER2+ breast cancer patients either with BCBM at clinical diagnosis. We used the DEPArray™, a new image-based CTC platform capable of isolating viable CTCs on a cell per cell basis, the smallest functional unit of cancer. We will directly link DEPArray™-isolated EpCAM-negative CTC subsets, having Notch1 and HPSE expression and combinations thereof, to clinical BCBM. =We will perform Notch1 and HPSE gain/loss-of-expression studies using the 4 combinatorial Notch1/HPSE CTC subsets; and employing pINDUCER, a novel and potent inducible shRNA/cDNA lentivirus to regulate Notch1/HPSE gene expression and BCBM development. We aim to demonstrate that the expression of Notch1 and HPSE axis in CTC subsets is directly related to, and critical for, CTC-induced BCBM onset.

15. SUBJECT TERMS
Breast cancer brain metastasis (BCBM), Multi-parametric flow cytometry (FACS), Notch-1, Heparanase (HPSE), CTC subsets, mammospheres, lentiviral transduction, pINDUCER.

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1. INTRODUCTION:

Breast cancer brain metastasis (BCBM) represents the most devastating and feared consequence of breast cancer. BCBM is usually fatal and is increasing in frequency with occult brain metastasis becoming exceptionally common at autopsy (1). However, mechanisms underlying BCBM are understudied and remain largely unknown. For example, although Circulating Tumor Cells (CTCs) are "seeds" of intractable metastases (2-6), there is no knowledge of properties or functionalities of brain-colonizing CTCs that can predict and/or to prevent breast cancer brain metastasis in patients. Uncovering BCBM CTC phenotypes offers the potential to modify treatment by extending studies directly to human cancer. Single-cell CTC transcriptional profiling has indicated that CTCs isolated from breast cancer patients are very distinct from breast cancer cell lines that are widely used for drug discovery, a finding which raises the important issue of the suitability of these lines for late stage breast cancer therapy (6). Further, highly significant discordances of breast cancer biomarkers among CTCs and corresponding primary and metastatic breast cancers have been also observed (2-4). Deciphering CTCs, notably EpCAM-negative CTCs per our discoveries (10), along with their properties may lead to more effective clinical trials by testing CTC-associated markers rather than solely relying on primary tumor biomarkers for therapy selection.

KEYWORDS: Breast cancer brain metastasis (BCBM), Multi-parametric flow cytometry (FACS), Notch-1, Heparanase (HPSE), CTC subsets, mammospheres, lentiviral transduction, pINDUCER.

2. OVERALL PROJECT SUMMARY:

This report represents progress made for the first year of the Breast Cancer Breakthrough Award. We have made progress in tasks associated with the three specific aims of the proposal, as described below.

Aim 1. To determine effects of therapeutic inhibition of Notch1 and HPSE CTC markers on BCBM onset.

Perform in vitro cell adhesion, mammosphere formation, cell proliferation and invasion of HPSE/Notch1 CTCs isolated from patients diagnosed with BCBM.

The rationale for studies proposed in this aim was to validate that CTCs possessing Notch1 and HPSE are the circulating cancer-initiating cells responsible for BCBM; and to link the respective combinatorial CTC subsets, to clinical BCBM. To this end, we isolated CTC subsets from blood of HER2+ metastatic breast cancer (stage IV) patients with or without clinically diagnosed BCBM employing multiparametric flow cytometry (FACS; ARIA IIID system)(10). Selection markers were applied to FACS analyses of peripheral blood mononuclear cells (PBMCs) isolated from blood of these patients (Ficoll gradients). We obtained the distinct FACS selection of cells negative for CD45 (the common marker for normal PBMCs/lymphocytes), EpCAM (epithelial cell adhesion molecule which is expressed in epithelial carcinomas and marker used by the CellSearch platform)(2-4), and for mesenchymal stem/circulating endothelial cell markers (CD105/CD73/CD90 and CD31/CD34/CD14, respectively); however, possessing positivity for cytokeratins (CKs), the stem-cell signature CD44+/CD24-, and for combinatorial expression of Notch1/HPSE (four CTC subsets/patient: Notch-1+/HPSE+, Notch-1-/HPSE+, etc.). Moreover, we confirmed that CTC subsets were putative CTCs by breast cancer genetic array profiling as well as by performing Small Tandem Repeat (STR) DNA fingerprinting analyses, latter to verify the distinction of patient-derived CTCs from cancer cell lines.

Second, to interrogate proliferation/mammosphere formation/adhesion/invasion properties of isolated EpCAM-negative Notch1/HPSE CTCs, we investigated the *in vitro* 3D CTC mammospheres formation by performing 3D-tumorsphere assays (12-14). We observed that Notch1 and HPSE combinatorial

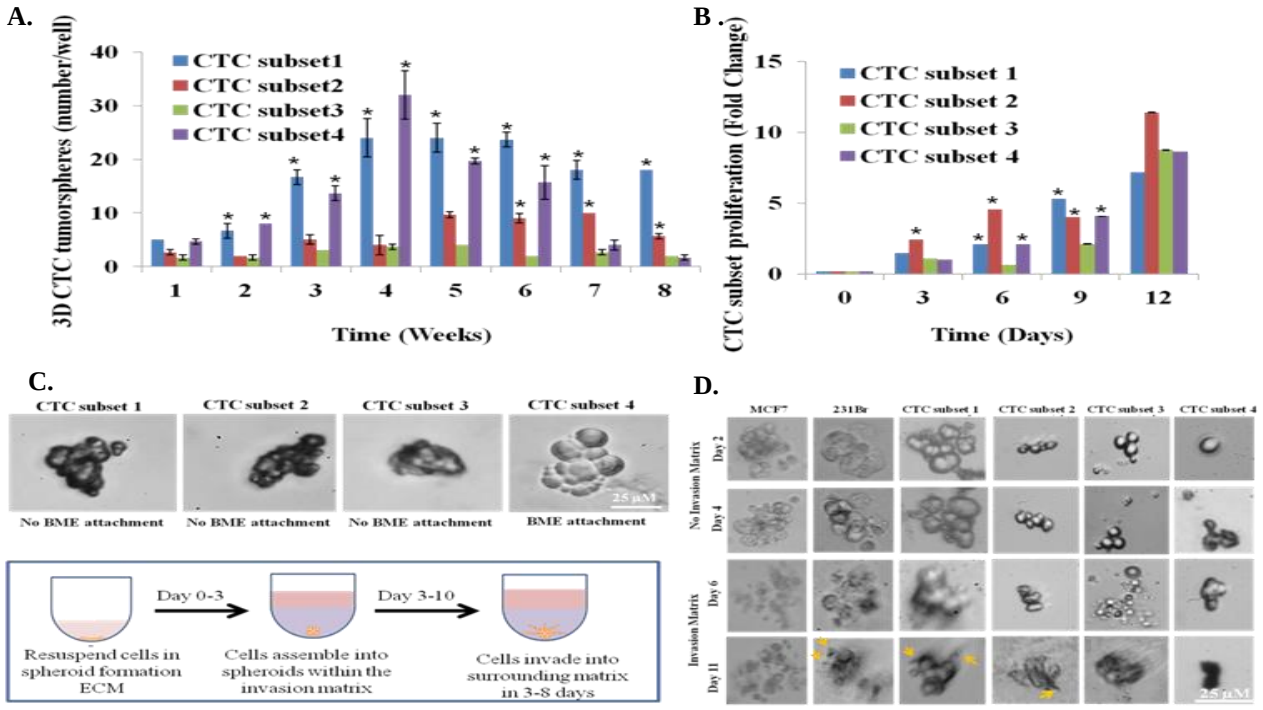

Figure 1. *In vitro* characterization of Notch1/HPSE CTCs (A.) Mammosphere assays were performed in FACS-sorted ($CD45^-/CD44^+/CD24^-$/EpCAM-negative/$Notch1^{+/-}/HPSE^{+/-}$) *in vitro* 3D CTC subsets derived from a BCBM patient. Trypsinized 3D CTC mammospheres were cultured in 96-well plate coated with 1% soft agar and quantified at successive weeks under phase contrast microscopy (Zeiss, Inc.). Blue: Notch1-/HPSE+. Red: Notch1+/HPSE-. Green: Notch1-/HPSE-. Purple: Notch1+/HPSE+ CTCs. **(B.)** CTC proliferation assays (WST-1, Roche Life Sciences, Inc.) were performed in FACS-sorted *in vitro* 3D CTC subsets containing Notch1/HPSE combinatorial expression. Trypsinized 10-15 3D CTC tumorspheres were cultured in 96-well plate coated with 1% soft agar. Absorbance was measured at 450 nm and 690 nm wavelength at 8 hrs after adding WST-1 reagent at different time points. All data are representative of at least three independent experiments. Mean standard deviation (±) were incorporated. Student paired type *2 t*-test were calculated and *p*-value* (<0.01) were calculated as significant; **(C.)** Four CTC subsets with combinatorial expression of Notch1 and HPSE were aliquoted into 96 well flat-bottom plates coated with Trevigen® PathClear Basement Membrane Extract® (BME) and incubated for 96 hours at 37^o C for adhesion assays; **(D.)** Four CTC subset-generated mammospheres were trypsinized and dissociated as single CTC units or pairlet cells (assay steps comprising the 3D cell culture 96 well BME cell invasion assays are shown on the left). Control, non-invasive/non-BCBM generating MCF7 and invasive/BCBM generating MDA-MB231BR breast cancer cells were trypsinized and cell viability was confirmed. Images were captured at endpoint under 40 X magnification using phase contrast microscopy (Zeiss, Inc.). Representative images of three independent experiments are shown.

expression with the four EcCAM-negative CTC subsets (CTC subset 1/2/3/4) expanded in size and number to cluster and to generate 3D CTC mammospheres. Distinct bell-shaped *in vitro* growth patterns were noticeable up to 10-week analysis endpoint **(Figure 1A)**. Of note, Notch1^{+}/HPSE^{-} CTC subsets generated 3D CTC macro-mammospheres (>5 cells) compared to CTC micro-mammospheres (<5 cells) of Notch1^{+}/HPSE^{+}, Notch1^{+}/HPSE^{-} and Notch1^{-}/HPSE^{+} subsets. Conversely, Notch1^{-}/HPSE^{-} CTC subsets showed delayed clustering and formation of 3D CTC mammospheres, independent of tumorsphere size **(Figure 1A)**. Spatial and temporal kinetics of *in vitro* CTC mammospheres from patient-derived, FACS-selected CTC subsets were also evaluated for reproducibility/CTC subset and visualized by phase-contrast microscopy **(Figure 2)**.

Third, we assessed the proliferative, adhesive and invasive capacities of patient-derived EpCAM-negative Notch1/HPSE CTC subsets. Cell proliferation assays applying 3D non-adherent cells methodologies to 3D CTC mammospheres revealed that these subsets possessed differential *in vitro* proliferation abilities that correlated with the combinatorial expression of Notch1 and HPSE markers. Further, Notch1^{+}/HPSE^{-} and Notch1^{-}/HPSE^{+} CTC mammospheres showed an additive proliferative capacity between days 9 and 12 **(Figure 1B)**.

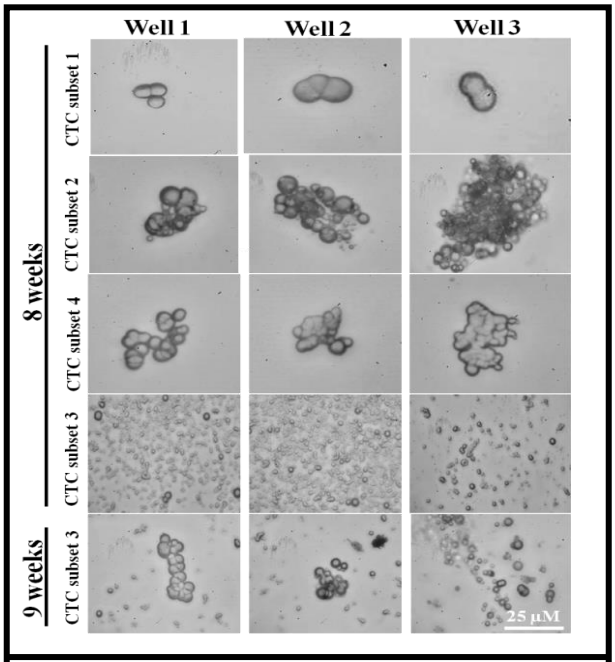

Figure 2. Spatial and temporal kinetics of *in vitro* 3D CTC mammoshperes. FACS-sorted EpCAM-negative/CD45-negative but CD44^{+}/CD24^{-}/Notch1$^{+/-}$/HPSE$^{+/-}$ CTC subsets were cultured *in vitro* to generate 3D CTC mammospheres derived from breast cancer patients. 3D CTC mammospheres (~10-15 clusters) were cultured on 1% soft agar coated 96-well plate. Images shown were taken for multiple wells for reproducibility, and at successive weeks using phase contrast microscopy (Zeiss, Inc.).

Fourth, to evaluate CTC subsets adhesion capabilities, we grew those using 3-D basement membrane extract (BME) tumorsphere assays (Trevigen® Inc.)(13, 14). We observed high adhesion of Notch1^{-}/HPSE^{+} CTC mammospheres on BME matrix at 48 hrs while the other three CTC subsets showed no attachment in adhesion assays even up to 96 hours incubation time **(Figure 1C).** Cell migration and invasion are fundamental processes which regulate important cellular events such as angiogenesis, invasion and metastasis of cancer cells. Interestingly, EpCAM-negative CTC subsets aggregated and formed *in vitro* 3D CTC mammospheres. Accordingly, we determined how CTC mammospheres generate invadopodia under well-controlled *in vitro* conditions, and abilities to become motile and to invade into extracellular matrix (ECM) of the 3D-invasion assay **(Figure 1D)** (12-14). Further, invadopodia formation by invading CTCs recapitulates the early steps of brain colonization observed *in vivo* (15). We assessed Trevigen® 3D tumorsphere invasion assays on *in vitro* 3D CTC mammospheres and visualized invadopodia formation. We used non-invasive poorly metastatic MCF7 and highly metastatic MDA-MB231BR breast cancer cells as negative and positive controls, respectively. We processed invasion matrix to monitor invadopodia formation at day 4 per assay conditions (12-14). Non-invasive control MCF7 cell-derived spheroids did not form any protrusions whereas invadopodia formation was noted when employing invasive 231BR spheroids. Of note, protrusions and tiny ruffle-like invadopodia were

observed in Notch1^{+}/HPSE^{-} and Notch1^{+}/HPSE^{+} CTC subsets at day 11 **(Figure 1D, yellow arrows)**. Conversely, no invadopodia formation was observed in Notch1^{-}/HPSE^{-} and Notch1^{-}/HPSE^{+} 3D CTC subset spheroids plated on BME invasion matrix per assay specifications (13, 14). These results demonstrate that the Notch1/HPSE biomarker axis enables invadopodia formation at distinct Notch1/HPSE CTC combinations (highest for Notch1+/HPSE+ CTCs) when subjected to the proper tumor microenvironment and factors. These findings are of relevance because the formation of invadopodia in CTC is required for the *in vivo* extravasation through blood-brain barrier as the early step toward CTC colonization of brain and BCBM development (15).

Transduce isolated Notch1/HPSE CTCs with luciferase lentivirus and/or enhanced green fluorescent protein (eGFP). Inject transduced CTCs into immunocompromised animals to recapitulate the metastatic cascade leading to BCBM onset. Monitor the development of BCBM in mice, per CTC subset and Notch1/HPSE expression.

Addressing these tasks and first, we labeled FACS-sorted and cultured Notch1/HPSE CTC subsets with eGFP-luciferase double fusion pCDH-MSCV-LUC2-EF1-GFP-T2A-Puro plasmid which was generously provided by Dr Joseph C Wu (Stanford University, CA). We performed lentiviral production and transduction employing lipofectamine 2000 (Life Technologies), psPAX2 (Addgene, plasmid 12260), and pMD2.G (Addgene, plasmid 12259). Second, we carried out experimental metastasis assays by injecting transduced CTC subsets into mice. Four-weeks old female SCID mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ strain; Jackson Laboratories; n=3/CTC subset) were injected either intracardiacally or orthotopically (fat pad injections) with CTC subsets. Cells were mixed with 100µl of 1:1 PBS and Matrigel (BD Biosciences). FACS-sorted CTC subsets not labelled with eGFP/luciferase (controls) were injected at intracardiac site and tumor growth was monitored every 9 days. Estrogen pellet (0.72mg; 90 day release; Innovative Research of America)(16) was subcutaneously implanted behind the neck of each mouse. *In vivo* animal imaging was subsequently performed after 20-30 min of intraperitoneal administration of D-Luciferase substrate (Gold Biotechnology) and using the IVIS (Xenogen) bioluminescent imaging system. We obtained contrasting results: while we were able to detect metastatic colonization by the Notch1-/HPSE+ CTC subsets, notably to brain **(Figure 3)**, we have not been able to reflect a similar behavior employing orthotopic injections of these CTC subsets, and independent of their isolation or patient origin (also note that no BCBM was detected using the MDA-MB231Br clone)**(Figure 4)**. There can be multiple overlapping reasons for this inability of CTC subsets to metastasize in mice. One is the low number of CTCs injected in these animals. We are presently addressing this issue by optimizing CTC growth conditions to obtain higher numbers of cultured CTC subsets to be injected into animals. A second reason can be the difficulty for CTCs to intravasate into the blood stream in these animals, an outcome that has been experienced by others (16; also, communication from Dr. Daniel Haber, MGH Cancer Center/Harvard Medical School). A third reason can be that these CTC subsets loose stem cell properties by long-term tissue culturing, thus key abilities to intravasate and to generate metastasis. We are currently addressing these issues by

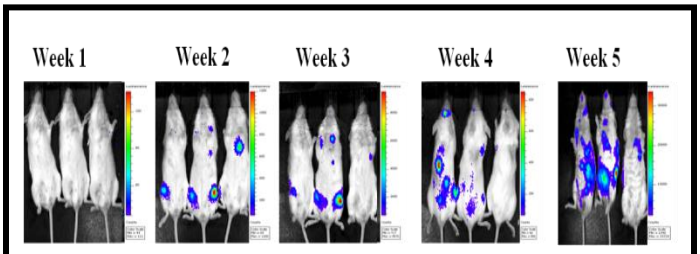

Figure 3. Animals (SCID/Beige mice) were injected at intracardiac site with eGFP-Luciferase labeled Notch1-/HPSE+ CTCs (5.0 x 10^5 cells in PBS/animal), in conjunction with a 90-day release estrogen pellet (Innovative Research of America) provided subcutaneously. Tumors growth was monitored every week by bioluminescent imaging using the IVIS (Xenogen) . Extensive metastasis formation, including BCBM was observed particularly at weeks 4-5 following CTC injections.

isolating Notch1/HPSE CTCs from patients' blood (FACS-isolated) and either injecting them directly into animals immediately following FACS isolation or culturing CTC subsets no longer than one month in tissue culture before animal injection not to loose important stem-cell properties.

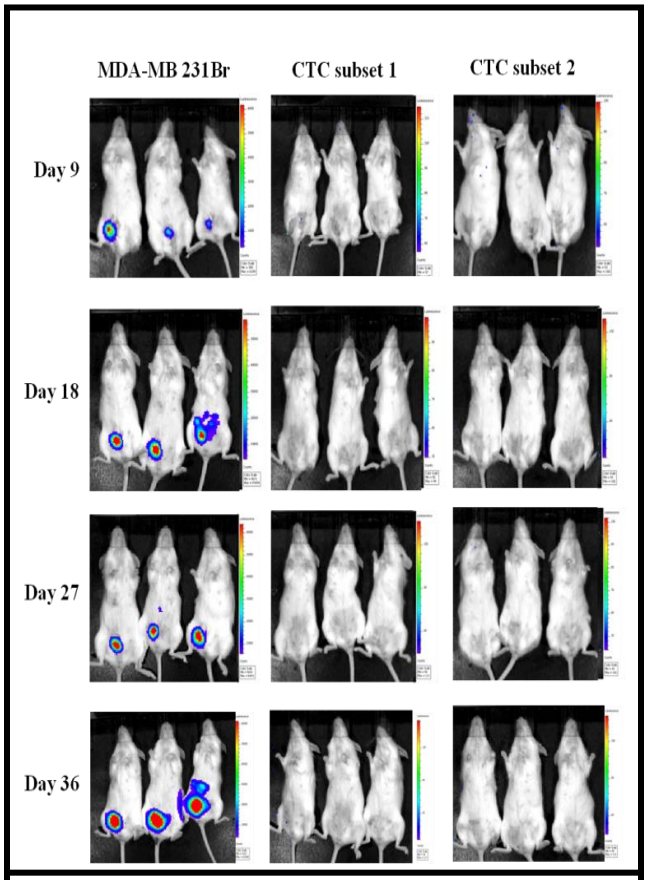

Figure 4. Animals (SCID/*Beige* mice) were injected orthotopically at mammary fat pad site with eGFP-Luciferase labeled CTCs and MDA-MB231BR cells (5 x 10^3 in PBS) with a 90-day release estrogen pellet (Innovative Research of America) which was provided subcutaneously. Tumors growth was monitored every 9 days by bioluminescent imaging using IVIS (Xenogen) instrument. No tumor formation by replicate CTC subsets was detected/cell injections/time.

Aim 2. To link combinatorial Notch1 and HPSE CTC subsets to clinical BCBM.

Capture and isolate CTCs, and perform CTC profiling to identify CTC subsets possessing HPSE and Notch1 and combinations thereof using the DEPArray platform.

We have isolated viable CTC subsets (aim 1) and compared the expression of Notch1/HPSE combinations from metastatic HER2+ breast cancer patients employing the novel DEPArray™ CTC platform. The DEPArray™ platform (Silicon Biosystems, Inc.) is a cell image-based microarray for the detection and unbiased recovery of specific CTC subsets and at single-cell level, thus capturing the smallest functional unit of cancer, e.g., single CTCs.

We have established the feasibility of FACS/DEPArray™ procedures and isolated EpCAM-negative, Notch1/HPSE-expressing CTC subsets from 5 HER2+ patients clinically diagnosed with BCBM**.** The CTC subsets proliferated in vitro allowing to retrieve cell quantities for downstream interrogation. FACS-isolated CTC subsets were sorted per DEPArray™ specifications (*all-or-none threshold for marker expression*). FACS-isolated EpCAM-negative and Notch1/HPSE CTC subsets were sorted per DEPArray™ specifications and were interrogated at the single-cell level.

We were able to not only confirm presence of the brain-metastasis-selected-marker profile (BMSM; 10) in these CTC subsets but also discover CTC subsets with a distinct and combinatorial presence of Notch1 and HPSE. This is of relevance since these CTC subsets will be important tools to interrogate the validity of CTC Notch1/HPSE and according to CTC-induced BCBM onset **(Figure 5).**

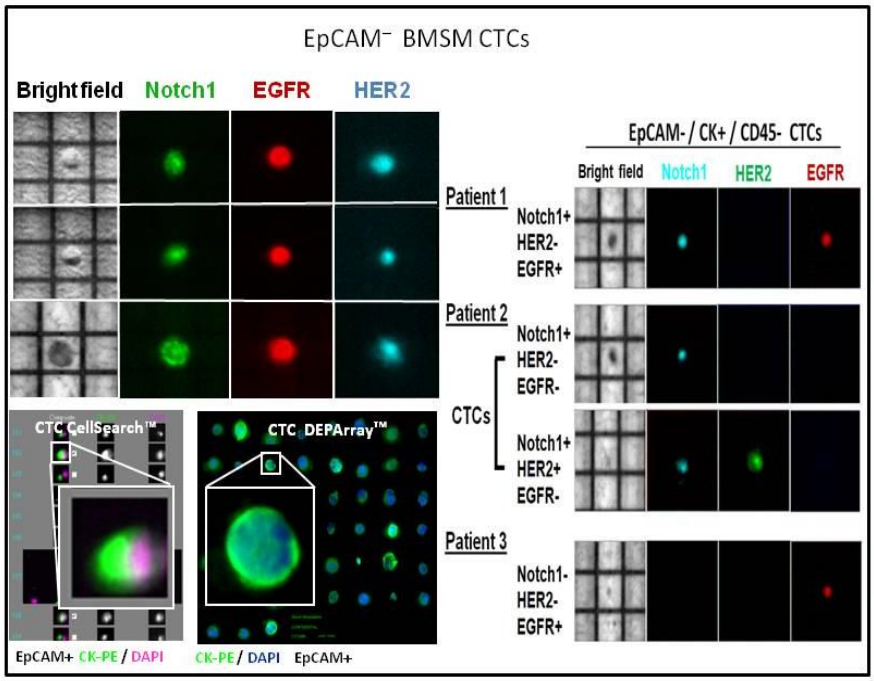

Figure 5. The isolation and characterization of single CTCs captured from HER2+ breast cancer patients by the DEPArray platform. **(Top left panel).** The isolation of EpCAM-negative CTCs by DEPArray (single-cell level capture and visualization by brightfield microscopy and immunofluorescence staining) possessing the Brain Metastasis Selected Marker (BMSM) CTC profile: Notch1 (FITC), EGFR (PE), and HER2 (APC). Shown are representative images of CTCs isolated from a HER2+ patient diagnosed with BCBM. **(Right panel).** The detection of specific EpCAM-negative CTC subsets by DEPArray possessing alternative combinations of markers of the BMSM CTC profile, including Notch1 (Notch1 positive or Notch1-negative CTC subsets). Combinatorial HPSE expression was confirmed by performing HPSE activity assays in these EpCAM-negative CTC subsets. Shown are representative images of EpCAM-negative/CK+/CD45-negative CTC subsets isolated from three HER2+ breast cancer patients diagnosed with BCBM: Notch1 (APC), HER2 (FITC), and EGFR (PE). (**Bottom left panel).** Visualization and morphology of CTCs captured by the DEPArray and FDA-cleared CellSearch. A reliable comparison was detected between the two platforms when EpCAM was chosen as cell surface antigen for CTC selection (CellSearch captures only EpCAM+ CTCs).

Aim 3. To assess the regulation of Notch1 and HPSE axis expression in CTC subsets that affect BCBM onset.

Implement the use of pINDUCER lentiviral tool kit by Notch/HPSE shRNA cloning and controls.
Implement the use of pINDUCER lentiviral tool kit by Notch/HPSE cDNA cloning and controls.

Overall objectives of this aim were to perform Notch1 and HPSE gain-/loss-of-expression studies using patient-isolated CTC subsets and employing pINDUCER, a novel inducible shRNA/cDNA lentivirus, to regulate Notch1 and HPSE gene expression and BCBM onset.

As first step, to assess the regulation of Notch1/HPSE expression in pINDUCER-treated CTC subsets, we initiated studies to construct these Notch1/HPSE pINDUCER lentiviral vectors. Specifically, we employed the pINDUCER11-mirRG inducible lentiviral gene silencing vector. Its insert, PheSGly294 (XhoI to EcoRI: 1.4 kb), can be replaced with the shRNA of a gene of interest in a mir30–based hairpin. To fit this criterion, the 21mer shRNA clones for Notch1 and HPSE predicted by The RNAi Consortium (TRC) were selected, and the available constructs from Sigma-Aldrich, Inc. (Dharmacon, owned by GE Healthcare and Broad Institute of MIT and Harvard) were evaluated. Moreover, the HPSE shRNA library, Notch1 shRNA library, GAPDH as well as non-silencing shRNAs were all adopted from GIPZ lentiviral shRNA library designed by Dharmacon/GE Open Biosystems. A schematic of pINDUCER vector contruction is shown in **Figure 6**.

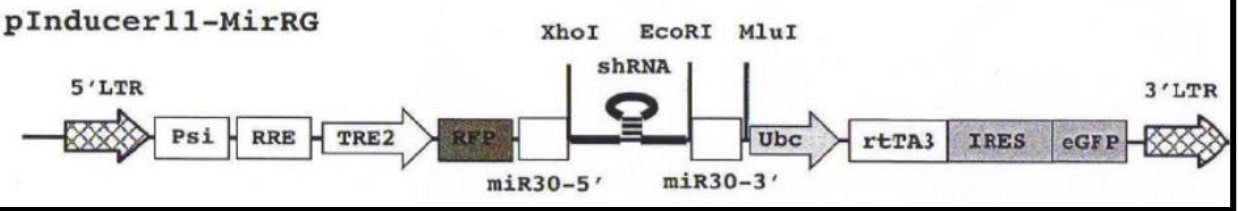

Figure 6. Hairpin structure pINDUCER11-MirRG is a lentiviral gene silencing vector. Four HPSE shRNA clones, four Notch1 shRNA clones, and two positive/negative controls (GAPDH and non-silencing shRNA, respectively) were bioinformatically examined to fit the miR30-based hairpin structure.

Because there were 10 pINDUCER11-shRNA plasmids to be constructed (4 for Notch1, 4 for HPSE, 1 for GAPDH as a positive control and 1 for non-silencing shRNA as a negative control), we considered important to apply a comprehensive procedure to simultaneously prepare insert shRNAs. Accordingly, per mir30 structure, we established an optimal PCR procedure including employing the forward primer –CAGAAGGCTCGAGTGCTGTTGACAGTGAGCG and the reverse primer –GCCCCTTGAATTCTCC GAGGCAGTAGGCA to generate XhoI and EcoRI sites in both ends; along with a two-step PCR program: 1) 95°C for 30 seconds, 2) 94°C for 10 seconds, 3) 72°C for 30 seconds, repeating 34 times steps 2 and 3 (35 cycles) with a final extension for 2 minutes at 72°C. We were successful to implement these strategies. Our results show that not only the small-size DNA template (97 bp) could be amplified but also high-quantity of product could be obtained **(Figure 7)**.

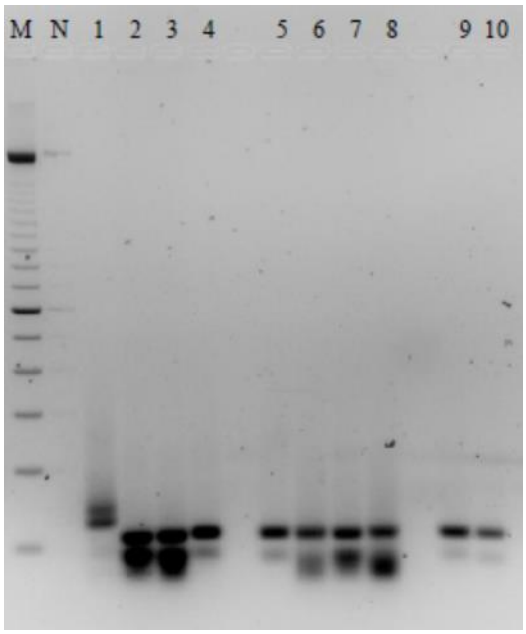

Figure 7. Preparation of shRNA employing optimal PCR conditions. An universal primer set and program were established to amplify a 97-base hairpin template. M: 100 bp DNA ladder (ThermalFisher Scientific); N: no-template control; lanes 1-4: four HPSE shRNA clones; lanes 5-8: four Notch1 shRNA clones; lane 9: GAPDH shRNA clone (positive control); lane 10: non-silencing shRNA clone (negative control).

Second, because one of the key elements in molecular cloning is the size of vector and its insert, the technical challenge was that vector pINDUCER11-mirRG (14699 bp) is substantially larger than the shRNA insert (109 bp). Although pINDUCER11-mirRG were digested with XhoI and EcoRI to generate the sticky end, the tendency of self-ligation cannot be avoided. Therefore, we applied the alkaline phosphatase from calf intestinal (CIP) methodology to raise the successful rate of pINDUCER11-mirRG and shRNA ligation. Following ampicillin selection, colonies were screened by EcoRI and XhoI double digestion and the sequence from positive colonies was verified by employing the primer, GTATCAAAGAGATAGCAAGGTATTC. Results are shown in **Figure 8**.

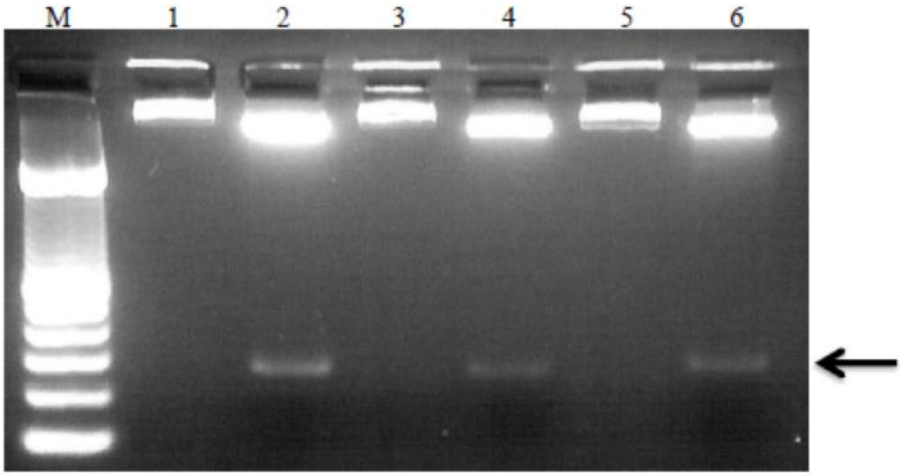

Figure 8. RT-PCR analyses showing the construction of pINDUCER11-shRNA plasmids. HPSE, Notch1, and non-silencing shRNA clones were cloned into pINDUCER11-MirRG vector, as shown. M: 100 bp DNA ladder (ThermalFisher Scientific); lane 1: pINDUCER11-HPSE shRNA plasmid; lane 2: pINDUCER11-HPSE shRNA plasmid doubly digested with MluI and XhoI; lane 3: pINDUCER11-Notch 1 shRNA plasmid; lane 4: pINDUCER11-Notch1 shRNA plasmid double digested with MluI and XhoI; lane 5: pINDUCER-non-silencing shRNA plasmid; lane 6; pINDUCER11-non-silencing shRNA plasmid doubly digested with MluI and XhoI. Arrow indicates the expected 109 bp insert size.

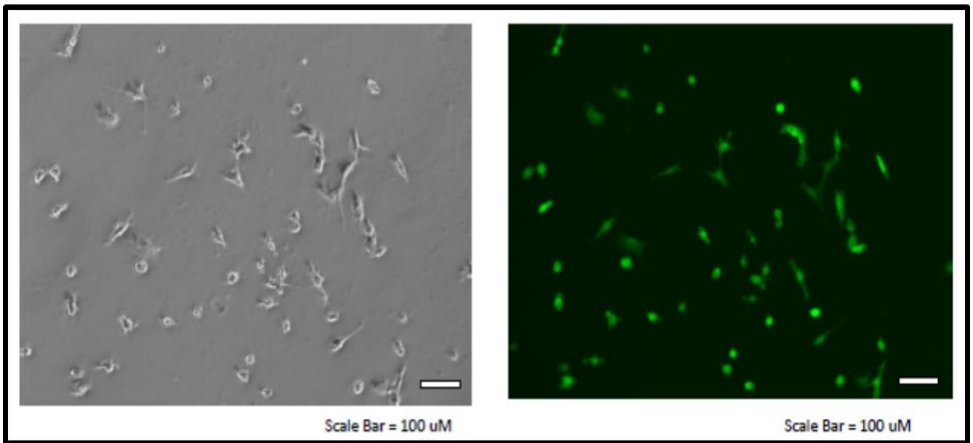

Figure 9. Immunofluorescence microscopy staining (left panel) showing that pINDUCER11–HPSE shRNA lentivirus produced by HEK–293T cells is able to infect human brain metastatic MDA–MB–231BR cells as inducible cell line. Left panel refers to corresponding phase contrast microscopy of these cells (control).

Third, to gain insights into the molecular mechanisms of HPSE and Notch1, an effective and long-term gene silencing in mammalian cell lines was generated. Of note, and considering that Notch1 and HPSE gene suppression may result in cell lethality, along with potential toxicity by the constitutively expressed shRNA. We selected an inducible shRNA system to circumvent these problems. Constructed plasmids were assessed by packing it into HEK–293T cells to produce virus followed by cell transduction. We achieved a successful of integration of this genome. Results of a constructed representative vector, namely pINDUCER11-HPSE shRNA, are shown in **Figure 9**.

Concluding, although there were distinct gene Notch1/HPSE shRNA libraries (4 clones for HPSE and 4 clones for Notch1) were selected to generate Notch1/HPSE gene silencing plasmids, one PCR was sufficient to prepare all shRNA inserts. The technical issue of cloning

was overcome considering that the parental vector, pINDUCER11-mirRG (14,699 bp), is ~143 times larger than its insert, shRNA (103 bp). Lentiviral particles produced from pINDUCER11-shRNA plasmids could successfully integrate into the genome of target cells, eg, human breast cancer cells competent for brain metastasis. This is an important achievement to perform Notch1/HPSE loss-of-function genetic studies in patient-derived CTCs monitoring BCBM onset.

4. KEY RESEARCH ACCOMPLISHMENTS:

1) Isolation of EpCAM-negative CTC subsets from peripheral blood of patients diagnosed with breast cancer brain metastasis (BCBM) possessing a combinatorial expression of Notch1 and HPSE (CTC subset 1/2/3/4, respectively);

2) Generation of 3D CTC-derived mammospheres by *in vitro* culturing and CTC mammospheres interrogation for biological characteristics and properties related to metastasis, e.g., adhesion, proliferation, invasion;

3) Capture of breast cancer patient-derived and FACS-isolated CTC subsets using the DEPArray platform, confirming presence of the BMSM CTC profile at the single-cell level;

4) Discovery of CTC subsets from BCBM patients possessing combinatorial expression for Notch1 and HPSE and their interrogation at single-cell level (DEPArray);

5) Optimization of PCR conditions to prepare distinct Notch1/HPSE shRNAs with the establishment of an effective cloning protocol to construct pINDUCER11-shRNA plasmids;

6) Construction of pINDUCER inducible lentiviral vectors for their delivery to mammalian cells for future assessment of Notch1/HPSE loss/gain-of-function studies in CTC subsets affecting their metastatic propensities.

5. CONCLUSION:

We isolated CTC subsets from peripheral blood of HER2+ patients diagnosed with breast cancer brain metastasis (BCBM). CTC subsets were selected for EpCAM negativity but positivity for CKs and $CD44^{+}/CD24^{-}$ stem cell signature; along with combinatorial expression of Notch1 and HPSE. These are markers directly implicated in breast cancer mechanisms (9,10), notably to brain per hypothesis put forward. CTC subsets were confirmed to be putative CTCs and were cultured *in vitro* generating 3D CTC mammospheres, Further, CTC-derived mammospheres were interrogated for biological characteristics and *in vitro* properties related to metastasis. We identified distinct proliferative, adhesive and invasive properties of 3D CTC mammospheres upon Notch1/HPSE expression in the four combinatorial Notch1/HPSE CTC subsets. Moreover, we interrogated breast cancer patient-derived and FACS-sorted CTC subset using the DEPArray CTC platform confirming the presence of the BMSM CTC profile, notably Notch1 and HPSE, at the single CTC level. Lastly, we made progress towards the construction of pINDUCER lentiviral vectors for their delivery to CTCs to assess Notch1/HPSE loss/gain-of-function studies in CTC subsets associating with BCBM onset in xenograft models. Although there were different gene shRNA libraries (4 clones for notch1 and 4 clones for HPSE) to generate the gene silencing plasmids, we established one PCR to be sufficient to prepare all

shRNA inserts. We also overcome technical cloning issues for the correct production of pINDUCER11-shRNA-mirRG inducible lentivitral vectors and their integration into the genome of target cells for future Notch1/HPSE loss-of-function genetic studies in patient-derived CTCs.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

None to report

7. INVENTIONS, PATENTS AND LICENSES:

None to report.

8. REPORTABLE OUTCOMES:

None to report.

9. OTHER ACHIEVEMENTS:

None to report.

10. REFERENCES:

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11. APPENDICES:

None.